Chapter I

Introduction & Review of Literature
INTRODUCTION

Stroke, the brain equivalent of heart attack is the second most common cause of death and long-term disability worldwide. Stroke is a major health problem which often affects the activities of daily life and cerebral function (Macrez et al., 2011). According to World Health Organisation (W.H.O), annually 15 million people worldwide suffer a stroke. Of these, 5 million die and another 5 million are left permanently disabled (Mackay and Mensah, 2004). Clinical features of stroke include hemiplegia, movement disorders, somatosensory impairments, spatial neglect, learning and memory, and tactile extinction (Cenci et al., 2002). Due to its huge socioeconomic burden, and with overall increases in life expectancy, one can predict that stroke will continue to be a tremendously challenging disease (Durukan and Tatlisumak, 2007). In humans, there are two common types of stroke: global that is induced by a total loss of blood flow to the brain, such as during a cardiac arrest, and focal which results in a localized infarction (Chiang et al., 2011; Hunter et al., 1995). Approximately 85% of all strokes are ischemic, which results from the sudden interruption of Cerebral blood flow (CBF), results from thrombolic or embolic occlusion of a major cerebral artery, most often the middle cerebral artery (MCA) or its branches (Chiang et al., 2011). The remaining 15% are hemorrhagic stroke, the most lethal type which results from a burst blood vessel in the brain or on its surface (Durukan and Tatlisumak, 2007).

The primary insult that ischemia brings to all brain cells is loss of the energy substrates: glucose and oxygen, which slows or stops the synthesis of ATP through glycolysis and oxidative phosphorylation (Sims et al., 2010; Yang et al., 2000). Oxygen is an irreplaceable driver of mitochondria respiration, the main source of cellular ATP. Therefore, lack of oxygen will reduce cellular ATP production, which results in a rapid decrease in cellular ATP concentration because of ongoing consumption (Li et al., 1995). When respiration is inhibited but glycolysis persists, protons and lactate generated during glycolysis accumulate, causing rapid intracellular acidification (from pH 7.3 to 6.5) (Choi et al., 1996; Riley et al., 1992). One important event that occurs during brain ischemia is the inhibition of Na⁺-K⁺ ATPase upon loss of cellular ATP (Tzen et al., 2007; Ikeda et al., 2002). Consequently, inhibition of Na⁺-K⁺ ATPase will lead to loss of ionic gradients and membrane depolarization in both neurons and surrounding cells like astrocytes.
The dissipation of ionic gradients is associated with a large increase in the extracellular concentration of glutamate (from 1 μM to 100-300 μM) and other neurotransmitters (Thimmulappa et al., 2002). The release of glutamate initiates a positive feedback loop, with the activation of glutamate receptors further decreasing ionic gradients and consuming ATP, both of which will promote further release of glutamate (Go et al., 2008). These events culminate with a large increase in intracellular calcium concentration from 70 nM to 30 μM, which triggers the death of...
neurons and astrocytes by a process known as excitotoxicity (Nguyen et al., 2003). After transient ischemia the reperfusion period is characterized by a delayed rise in cytoplasmic free calcium (Liu et al., 2007). The influx of Ca\(^{2+}\) into cells is the most significant event in the pathogenesis of ischemic brain damage (Shah et al., 2007; Shih et al., 2005). Ca\(^{2+}\) mediated excitotoxicity mechanism is complex but primarily involves activation of proteases, generation of ROS, mitochondrial Ca\(^{2+}\) overload and mitochondrial dysfunction, resulting in immediate or delayed cell death (Wang et al., 2007) (Fig. 1.1).

The priority placed on developing strategies to treat stroke has prompted research aimed at understanding the cellular, behavioural and neurological underpinnings of ischemic brain injury. Even though enormous efforts have focused on the development of drugs to limit brain damage caused by acute ischemic stroke, there is as yet no routine, effective, generally accepted, specific treatment for acute ischemic stroke, except for aspirin (Tufano et al., 2011). Thrombolysis with tissue plasminogen activator is effective in highly selected patients (within three hours of stroke onset), but the data do not support the widespread use of thrombolytic therapy in routine clinical practice because of the high risk of bleeding (Bruce et al., 2011). Therefore, it is necessary to test other promising strategies such as neuroprotective agents.

Epidemiology
Currently, stroke ranks as the leading cause of adult disability with approximately one-third of patients who will survive at least 6 months after stroke being dependent on others (Warlow, 1998) and is estimated to be the second leading cause of death worldwide (Macrez et al., 2011). About one-sixth of all human beings suffer at least one stroke during their lifetime (Seshadri et al., 2006). This estimate breaks down to one person having a stroke every 40 seconds and one person dying from a stroke every 3 minutes (Rosamond et al., 2008).

It is estimated that about one third of stroke victims die; another one third are disabled permanently (Wolf and D’Agostino, 1980). Two-thirds of these fatal subjects occurred in the people living in the developed countries (W.H.O, 2002). The loss of these patients from the work force and the extended hospitalization they require during recovery make the economic impact of the disease one of the most devastating in medicine.
In India it has presently turned into a major public hazard with approximately 2 million patients per year, and the incidences of stroke is likely to increase in the coming year due to increase in population, life expectancy, stress level and changing life style involving smoking, excess alcohol use. The last available estimates from Indian Council of Medical Research (ICMR), Government of India indicate that in 2004 there were 930,985 cases of stroke in India with 639,455 deaths and 6.4 million disability adjusted life years (DALY) lost (Table. I).

Types of Stroke

Stroke is a heterogeneous injury involving disruption of blood flow to the brain. The resulting dysfunction or death of neural tissue creates neurological deficits that reflect loss of function by the compromised areas. The two general ways that blood flow is disrupted during stroke are: Hemorrhagic and ischemic (Fig.1.2). Hemorrhagic stroke occurs when a blood leaks from the cardiovascular system resulting in reduced blood flow. Hemorrhagic stroke has an intracerebral origin in 10% and a subarachnoid origin in 5% of all stroke cases (Lloyd-Jones et al., 2009). Ischemic stroke accounts for approximately 85% of all stroke types and is characterized by compromised
blood flow due to a blood vessel obstruction or an inability for the cardiovascular system to maintain adequate supply such as cardiac arrest (Lloyd-Jones et al., 2009).

**Classification of cerebral ischemia**

Cerebral ischemia can be grouped into two major categories:

A. **Global or forebrain ischemia**: It mimics the neuronal loss typically observed following coronary artery bypass or heart attack in humans (Hunter et al., 1998), and consist of a 5-15 minute cessation of cerebral blood flow, followed by reperfusion (Small & Buchan, 2000; Ginsberg, 2003). These models include bilateral occlusion of the common carotid arteries (two vessel occlusion), bilateral occlusion of the common carotid and vertebral arteries (four vessel occlusion), and decapitation (Ginsberg & Busto, 1989; Hossmann, 1991; Hunter et al., 1995; Small & Buchan, 2000).

B. **Focal ischemia** is a state of blood flow and nutrient suspension within the distribution area of a single artery (Wauquier et al., 1987) that leads to stroke, involves only part of the cerebral tissue and is often caused by middle cerebral artery (MCA) occlusion (Marco et al., 2010). In this type of ischemia it is easy to discriminate between the core tissue, an area with relatively dense ischemia, and the penumbra tissues, which are less dense due to their collateral blood supply from other major arteries (Siesjo et al., 1995) (Fig.1.3).

Focal ischemia can either be thrombotic or embolic in origin. Thrombotic focal ischemia occurs when the blockage is formed at the site where it disrupts blood flow whereas embolic focal ischemia occurs when the blockage is formed distant to the site where it disrupts blood flow and is then carried in the blood stream to the place of the blockage. An additional type of ischemic injury is a transient ischemic attack.
TIA’s are often considered mini-strokes, indicating temporary reductions in blood flow where neurological symptoms last less than 24 hours. The onset of a TIA is usually sudden, generally lasting between 2 and 30 minutes (Hankey, 1996). While TIA’s are highly transient, they tend to foreshadow upcoming ischemic embolism strokes that are typically more severe (Hankey, 1996).

It is important to note in both global and focal cerebral ischemia, the insult can be either complete or incomplete. Complete ischemia is caused by total cessation of perfusion within an organ or part via the specific vessels that supply blood to the tissue (Bacigaluppi et al., 2010). On the other hand, incomplete perfusion can be seen as a continuum between normal flow and a total absence of flow that can distinguish tissues into three distinct parts.

ARTERIES TO THE BRAIN
A high rate of oxidative metabolism is characteristic for the brain. Under normal conditions, the adult brain consumes 3-4 ml O₂/min per 100 g tissue, and this can represent up to 20% of all inhaled oxygen. When collateral cerebral circulation (Table II) is altered, oxygen consumption in the ischemic zone is dramatically reduced, thus inducing significant neuronal injury (Kaushal and Schlichter, 2008; Sims and Zaidan, 1995). There are two main arterial systems by which brain cells need a constant supply for their survival and healthy functioning:

A) Carotid artery  B) Basilar artery

**Carotid artery**
In human anatomy, the carotid artery is an artery that supplies the head and neck with oxygenated blood; it divides in the neck to form the external and internal carotid arteries.

**Basilar artery**
In human anatomy, the basilar artery is one of the arteries that supply the brain with oxygen-rich blood. The two vertebral arteries and the basilar artery are sometimes together called the vertebrobasilar system, which supplies blood to the posterior part of circle of Willis and anastomoses with blood supplied to the anterior part of the circle of Willis from the carotid arteries (Fig. 1.4).
### Table. II Collateral cerebral circulation of the brain (Adapted from Carreira et al., 2009)

<table>
<thead>
<tr>
<th>Intracranial</th>
<th>Arteries connected</th>
<th>Connecting arteries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circle of Willis</td>
<td>Internal carotid artery and basilar/ posterior cerebral artery</td>
<td>Posterior connected artery</td>
</tr>
<tr>
<td>Vertebrobasilar and circle of Willis</td>
<td>Internal carotid artery and Vertebral / basilar arteries</td>
<td>Anterior connected artery</td>
</tr>
<tr>
<td>Tectal plexus</td>
<td>Posterior cerebral artery and superior cerebellar artery</td>
<td>Trigeminal, otic and hypoglossal arteries</td>
</tr>
<tr>
<td>Central artery branches</td>
<td>Branches of middle, anterior and posterior cerebral arteries</td>
<td>Tectal rami, connecting supra and infratentorial arteries</td>
</tr>
<tr>
<td>Leptomeningial</td>
<td>Pial plexus Neighbouring branches of major cerebral arteries</td>
<td>Anastomoses of terminal branches within and between arterial territories</td>
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<tr>
<td></td>
<td>Meningeal Central and meningeal arteries</td>
<td>Arterial branches of same or adjacent arteries</td>
</tr>
<tr>
<td>Extracranial</td>
<td>Arteries connected</td>
<td>Connected arteries</td>
</tr>
<tr>
<td>Orbital plexus</td>
<td>Ophthalmic and middle meningeal, maxillary, ethmoidal arteries</td>
<td>Terminal branches</td>
</tr>
<tr>
<td>Rete mirabile caroticum</td>
<td>Internal and external carotid</td>
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![Fig.1.4 Cerebrovascular System shows Carotid and Basilar artery](image) (Adapted from Adam images).
Pathophysiology of Stroke

Cell death during ischemia is thought to largely arise from excitotoxicity and ionic imbalance, oxidative/nitrosative stress and activation of apoptotic pathway, however, emerging evidence also implicates other detrimental processes including inflammation, tissue acidosis and peri-infarct spreading depolarizations (Mergenthaler et al., 2004) (Fig. 1.5). During ischemic exposure cells may die by rupture, lysis, phagocytosis or involution and shrinkage (Lo et al., 2003). These modes of cell destruction are often classified using the terms necrosis and apoptosis to discriminate whether the cell actively participates in its destruction.

Fig. 1.5 Graphical representation of events in cerebral ischemia

Necrosis is the most prominent form of cell death during extreme ischemia and is the result of rapid disruption of the plasma membrane or organelle failure (Lo et al., 2003). Necrotic cells endanger neighboring cells due to the release of toxic or damaging molecules (Lo et al., 2003). In contrast apoptosis represents a programmed cell death requiring adenosine tri-phosphate and gene expression that involves an organized degradation of the cell such that there is minimal release of cellular contents into its surrounding environment (Johnson et al., 1995).
Ischemic stroke may also arise from the atherosclerotic large cerebral arteries (eg, carotid, middle cerebral and basilar arteries) or atherosclerotic small cerebral arteries (eg, lenticulostriate, basilar penetrating, and medullary arteries). In brain the stroke generally is a characteristic of large artery blockage rather than small arteries supplying deep cerebral white matter. Atherogenesis is a decades-long process in which the lumen of a blood vessel becomes narrowed by cellular and extracellular substances to the point of obstruction (Breslow, 1996, Pruissen et al., 2009) (Fig.1.6). It was seen that the earliest lesion of atherosclerosis is the fatty streak (Stary, 1989).

On microscopy, the lesions primarily consist of lipid-filled macrophages (foam cells).

**Arterial wall injury**

For arterial wall injury, the concept is that atherosclerosis begins as a response to chronic minimal injury to the endothelium (the continuous monolayer of cells lining the arterial wall) and those interactions among monocytes, lipoproteins, platelets, lymphocytes, and smooth muscle cells abet and continue the pathogenic process. The main "battleground" of the atherosclerotic process is the intima, which lies just below the endothelium. While the media (middle layer) appears to play some role and perhaps the adventitia (outer layer) as well the development of atherosclerosis is primarily characterized by an accumulation of complex lipids, proteins, and carbohydrates, as well as a proliferation of cells, in the intimal layer of an artery. Pathophysiologic classification of vascular injury divided into three types (Fig.1.7).

- A chronic minimal injury characterized by functional alterations of endothelial cells without significant morphologic changes (i.e. epithelial denudation), is thought to be caused primarily by the turbulence of blood flow.
- Injury is characterized by denuding of the endothelium and superficial intimal injury. i.e. injury of soft plaques, with thrombus incorporation, is thought to be a major mechanism for the progression of atherosclerosis.
Injury is typified by deep intimal and medial damage, accompanied by marked platelet aggregation and mural thrombosis.

**Role of Monocytes**

Adhesion of circulating monocytes (white blood cells that become particle ingesting macrophages once they enter another tissue) to the surface of intact endothelial cells appears to be an early event in the development of atherosclerotic lesions. Monocyte binding to the endothelium of animals fed a high cholesterol diet is preceded by expression of the vascular cell adhesion molecule (VCAM) (Gimbrone et al., 1995). Immune mechanisms also appear to play a role in atherogenesis (Ip et al, 1990; Pasceri et al., 2000). Lymphocytes (cells responsible for the cell mediated immune response) have been found to be present, albeit in small numbers, in both early fatty lesions and in advanced fibrous lesions in humans. As atherogenesis progresses, these macrophages take on a "foamy" appearance thus their designation as "foam cells" (Fig.1.8) and become one of the primary components of the fatty streak.

**Role of Platelets**

Platelet aggregation and thrombosis may be promoted by toxic products released by macrophages and by moderate damage to the intimal surface with denudation of the platelets release growth factors that stimulate migration and proliferation of smooth muscle cells also contribute to the formation of subendothelial "fibrointimal lesions" (Fig.1.9) and possibly to formation of the outside capsule of predominantly "fatty lesions".
Plaque fissuring and formation

Plaques in the coronary arteries that have undergone fissuring indicate that the majority are composed of eccentrically situated lipids (i.e., located in an area where the vessel bifurcates) that do not have an internal lattice of collagen supporting the cap of the plaque (Fig. 1.10). The vulnerability of such a structure to fissuring appears to be related to circumferential stress on the plaque cap in systole, as well as infiltration of the cap tissue with foam cells (with reduction of total collagen content and a concomitant fall in tensile strength) (Rekhter et al., 1998).

Fig. 1.10 Plaque fissure platelet thrombi moves to microvascular

Types of cell death during stroke

Blockage of a cerebral artery results in the interruption of the blood flow and supply of nutrients: glucose and oxygen to the brain. Blood flow levels are important in determining the size of the infarct by providing the conditions essential for maintenance of cellular energy hemostasis. Decreased blood flow leads to a reduction in phosphocreatinine and ATP. In prolonged cases of ischemia, the energy depletion will be sufficient to lead to severe impairment of cellular function by disruption of ATP dependent processes. The energy needs of the brain are supplied by metabolism of glucose and oxygen for the phosphorylation of ADP to ATP. Most of the ATP generated in the brain is utilized to maintain intracellular homeostasis and transmembrane ion gradients of sodium, potassium, and calcium. Energy failure results in collapse of ion gradients and excessive release of neurotransmitters such as dopamine and glutamate (Adibhatla and Hatcher, 2008). Certain features of cell death, such as loss of energy stores, mitochondrial failure, macromolecular breakdown, and free radical production are shared regardless of the pathway employed. Overall, the differences between the types of cell death are primarily attributed to the timing and the subcellular location of the death pathway initiated by the cell (Choi, 1996).
Introduction & Review of literature

Events leading to cell death

Oxidative stress

Reactive oxygen species (ROS) have recently been hypothesized to play a role in the coordinated mechanism of cellular signaling. They have been found to stimulate a number of signal transduction pathways that are important in maintaining cellular homeostasis in the neurons. The contribution of oxidative stress after ischemia-reperfusion injury can lead to a vicious cycle as it impinges upon mitochondrial dysfunction, excitotoxicity, lipid peroxidation, and inflammation (Crack and Taylor, 2005). Free radicals (highly reactive oxygen species characterized by a free electron) generated during ischemia can cause considerable damage to lipids, DNA, and proteins, all the while contributing to the process of neuronal death. Free radicals also contribute to the breakdown of the blood-brain barrier and brain edema. Activities of free radical scavenging enzymes (e.g., superoxide dismutase) decrease during ischemia and nitric oxide levels are elevated. Nitric oxide generated primarily by neuronal and inducible nitric oxide synthases promote neuronal damage after ischemia (Barone and Feuerstein, 1999). Several experimental studies suggest that Nitric oxide synthases (NOS) constitute an important source of free radicals. This enzyme produces nitric oxide (NO), which is then able to combine with superoxide anions to generate the strong oxidant peroxynitrite anions.

Free Radical Damage

The brain and nervous system are particularly vulnerable to free radical damage for a number of reasons. The membrane lipids in the brain contain high levels of polyunsaturated fatty acid side chains, which are prone to free radical attack, and are readily peroxidisable, contributing to structural and functional perturbations of the membrane and cell function. The brain also consumes large quantities of total oxygen for its relatively small weight, contributing further to the formation of reactive oxygen species. It has been estimated that up to 2% of the oxygen consumed by healthy mitochondria is converted to superoxide, and this amount is higher in damaged mitochondria.

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Reactive oxygen species

ROS derived from oxygen are the superoxide anion (’O2'), the perhydroxyl radical (protonated superoxide, HOO) the hydroxyl radical (HO), and free radical nitric oxide (NO'). The one electron reduction of oxygen (i.e., the removal of one electron from oxygen molecule) results in formation of ’O2' (also known as the superoxide radical), whereas the two-electron reduction product of oxygen, when fully protonated, forms hydrogen peroxide (H2O2). These oxygen radicals result direct reactions of superoxide (or its conjugate acid) with biological targets, such as lipids (Dix and Aikens, 1993), catecholamines (Macarthur et al., 2000 and Heikilla and Cohen, 1973), and DNA (Dix et al., 1996). The most reactive oxy radical is HO2 produced from the interaction of ’O2' and H2O2 by a chemical process known as the Habitat- Fentons reaction

\[ M^{n+} + H_2O_2 \rightarrow OH^- + 'O_2H + M^{(n+1)+} \]

Superoxide reaction

\[ 'O_2' + M^{(n+1)+} \rightarrow O_2 + M^{n+} \]

Haber-Weiss reaction

\[ 'O_2' + H_2O_2 \rightarrow OH^- + 'O_2H + O_2 \]

Weiss reaction and iron catalyzed Fenton reaction to produce the hydroxyl radical. Superoxide generation seems to be based on its direct reactivity with numerous types of biological molecules (lipid, DNA, RNA, catecholamines, steroids, etc.) and damages cell membrane.

Reactive nitrogen species

NO is synthesized from the guanidino group of L-Aginine by a family of enzymes termed NOS. Generation of NO and O2 favors the production of a toxic reaction product, peroxynitrite anion (ONOOO) (Beckman et al., 1990). It is reasonable to conclude that peroxynitrite overproduction may occur readily in vivo. Once near or inside a cell, ONOOO can damage or deplete a number of vital components e.g., DNA by strand scission (Sestili et al., 2000), lipids by peroxidation (Radi et al., 1991 and Rubbo et al., 1994) and antioxidant availability (Van der Vliet et al., 1994; Vasquez-Vivar et al., 1996).
Na⁺- K⁺ ATPase dysfunction

Sodium, potassium-adenosine 5'-triphosphatase (Na⁺- K⁺ ATPase) is an integral membrane enzyme that actively transports K⁺ and Na⁺ ions against the respective cellular concentration differences. The Na⁺-K⁺ ATPase uses energy derived from hydrolysis of ATP to pump Na⁺ out and K⁺ into the cell as shown in (Fig.1.11). The gradient produced by this enzyme is coupled to physiological functions such as cell proliferation, volume regulation, maintenance of the electrogenic potential required for the function of excitable tissues, i.e. muscle and nerves, and secondary active transport (Jørgensen, 1992; Boldyrev, 1993; Basavappa et al., 1998). By regulating sodium and potassium ion concentrations, this enzyme also participates in the control of plasma membrane and mitochondrial Na⁺/Ca²⁺ exchange, the endoplasmic reticulum and plasma membrane Ca²⁺-ATPase activity, as well as Ca²⁺-channel activity. All these events control the cellular Ca²⁺ level and influence heart and vascular muscle contractility and neuronal excitability (Jørgensen, 1992; Nikezic and Metlas, 1985; Stahl and Harris, 1986; Calabresi, 1999). This enzyme is a glyco-protein composed of two subunits, a catalytic α subunit involved in splitting of ATP and a β subunit. The catalytic subunit of Na⁺-K⁺ ATPase is expressed in various forms (1, 2 and 3) the proportions of which may differ in various tissues. The 3 isoform seems to show a lower affinity for intracellular Na⁺, seems to be higher in cells containing mainly this version of the Na⁺-K⁺ ATPase such as neuronal cells (Munzer et al., 1994). Na⁺-K⁺ ATPase supporting the ionic homeostasis of the cell is under control of Na⁺, K⁺, Mg²⁺ and ATP. Due to the great importance of Na⁺-K⁺ ATPase in the maintenance of neuronal resting membrane potential and propagation of neuronal impulses, the malfunction of this enzyme has been associated with neuronal hyper-excitability, cellular depolarization and swelling (Lees, 1991). Ischemia causes Na⁺-K⁺ ATPase failure through non-inactivating Na⁺ channels coupled with severe K⁺ depletion that results in large membrane depolarization, high [Na⁺], stimulates reverse Na⁺-Ca²⁺ exchange and axonal Ca²⁺ overload (Stys, 1998). K⁺ ATPase activity is decreased by
toxic actions of normal neurotransmitters such as glutamate (Brines et al., 1995), which is the cause of cell injury and death of neurons in cerebral ischemia.

Mitochondrial dysfunction
Mitochondria play important roles as the powerhouse of the cell. Their primary physiological function is to generate adenosine triphosphate through oxidative phosphorylation via the electron transport chain, which contains five multi-subunit enzyme complexes, I to V. Reactive oxygen species (ROS) are generated in complex I and complex III during mitochondrial respiration (Boveris and Chance, 2009). Therefore, mitochondrial oxygen metabolism can be a potential threat to tissues and cells.

Mitochondria manifest signs of outer membrane and/or inner membrane permeabilization when exposed to a variety of pro-apoptotic second messengers. Pro-apoptotic members of the Bcl-2 family (Bad), which bear resemblance to channel-forming bacterial toxins, induce mitochondrial membrane permeabilization when added to purified mitochondria as recombinant proteins. This has been shown for Bax, Bak and Bid, and probably reflects the principal mechanism by which these proteins trigger cell death (Kroemer and Reed, 2000). The mitochondria have been identified as targets of cytoprotection for several disease states including stroke, mainly due to the high susceptibility of the organelle to cellular insults (Mehta and Li, 2009). In the brain, the mitochondria are especially vulnerable due to the high metabolic activity of the brain that is key in producing significant levels of cell-damaging oxidants (Szeto, 2006a; Szeto, 2006b).

Apoptosis
The number of cells in a multicellular organism is tightly regulated from conception up until death. If cells are no longer needed, they are programmed to commit suicide by activating an intracellular death program. This process is called apoptosis. For example, apoptosis occurs during various stages of embryonic development. Mouse paws and human hands are sculpted by cell death during this stage. Cell death helps to regulate cell numbers. In the case of neuronal development, cell death adjusts the number of nerve cells to match the number of target cells that require innervation (Campbell et al., 1999).
Cells that undergo apoptosis appear to die neatly, without damaging their neighbors. Initially, the cell shrinks and condenses. Then, the cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear DNA breaks up into fragments. Most importantly, alterations in the cell surface induce the expression of properties that cause the dying cell to be rapidly phagocytosed, either by a neighbouring cell or by a macrophage before any leakage of its contents occur. Not only does this prevent the damaging consequences of cell necrosis but also allows the organic components of the dead cell to be recycled by the cell that ingests it.

The intracellular machinery responsible for setting the process of apoptosis into motion appears to be conserved between species. A family of proteases that cleave their target proteins are called caspases. Caspases are synthesized in the cell as inactive precursors, or procaspases, that are activated by cleavage at aspartic acids by other caspases. Once activated, caspases cleave in turn activating other procaspases, thus amplifying the proteolytic cascade. Some of the activated caspases then cleave other key proteins in the cell. Activation of the intracellular cell death pathway is usually triggered in a complete, all-or-none fashion. The protease cascade is not only destructive and self-amplifying but also irreversible, so that once a cell reaches a critical point along the path to destruction; it cannot turn back (Campbell et al., 1999).

Procaspase activation can also be triggered by adaptor proteins that induce aggregation of multiple copies of initiator procaspases. Some initiator procaspases have a small amount of protease activity that is amplified when brought into proximity with other procaspases, thus triggering activation via cleavage. Procaspase activation can also be triggered from outside the cell by the activation of death receptors on the cell surface.
The Fas pathway (Fas is a death receptor) is known to be involved in apoptosis after cerebral ischemia. mRNA and protein levels of both Fas and the Fas ligand are upregulated after cerebral ischemia (Crosby et al., 2007; Jin et al., 2001). Mutant mice that have a loss-of-function mutation for Fas show reduced infarct volume after focal cerebral ischemia. Fas, Fas-associated death domain, and procaspase-8 form a protein complex that is referred to as the death-inducing signaling complex (DISC). DISC activates procaspase-8, similar to procaspase-9 activation by the apoptosome. Caspase-8 activation is followed by activation of caspases-3 and caspase-10 after cerebral ischemia. Stressed or damaged cells initiate apoptosis by producing both the Fas ligand and the Fas protein, thereby triggering an intracellular caspase cascade. Alternatively, stressed/damaged cells can initiate apoptosis by triggering procaspase aggregation and activation within the cell (Campbell et al., 1999).

In response to cerebral ischemia, apoptosis can also occur when certain organelles within the cell release pro-apoptotic compounds. Specifically, the mitochondria have been highlighted as sensitive organelles that respond to ischemic cellular insults by releasing a pro-apoptotic protein known as cytochrome c. Once present in the cytosol, it binds and activates an adaptor protein called Apaf-1 that recruits procaspase-9 and activates them in a process similar to the activation cascade of
proteins can serve dual (and conflicting) roles within the cell when apoptosis is initiated. They may protect or mediate the apoptotic cascade by participating in more than one pathway. One of the major proteins involved in this dual-signaling is the Bcl-2 family of intracellular proteins. These proteins not only regulate procaspase activation and block the release of cytochrome c from the mitochondria; they can also promote procaspase activation and cell death. Proteins within this family can inhibit apoptotic activity (Bcl-2), promote the pro-apoptotic activities of other proteins within the family (Bid), or work solely in mediating apoptosis (Bax, Bak and Bid) depending on cellular signals (Campbell et al., 1999).

Crosstalk between the intrinsic pathway and the extrinsic pathway
There are two general pathways for activation of apoptosis: the intrinsic and extrinsic pathways (Fig. 1.13). Over the last decade, experimental studies have provided considerable new information characterizing apoptotic processes occurring after ischemic stroke.

The extrinsic pathway is the death-receptor-mediated pathway that receives extracellular signals and transduces them to intracellular signals. Recent studies have shown that the death receptor pathway has various physiological functions as well as apoptotic roles. The intrinsic apoptosis pathway mediates the caspases-dependent death of cells in response to intrinsic signals (Benn and Woolf, 2004; Meier and Vousden, 2007). This involves mitochondrial permeability transition (MPT) and release of apoptotic factors including cytochrome c and Smac/Diablo, controlled by the balance of pro- and anti-apoptotic members of the Bcl-2 superfamily (Youle and Strasser, 2008).

There is crosstalk between the intrinsic pathway and the extrinsic pathway. The key molecule involved in this crosstalk is Bid, which is also a key molecule for the p53-caspase-2 pathway. Bid is truncated by caspase-8, translocates to mitochondria, and
interacts with other Bcl-2 family proteins, which causes cytochrome c release followed by apoptotic cell death (Plesnila et al., 2001).

**Inflammation**

The pathophysiological role of acute inflammation in adult experimental stroke models has been demonstrated over the years and the relationships between the presence of reperfusion, gender, genetic background and the extent, timing and consequences of injury have been largely established (Iadecola and Alexander, 2011). The results of clinical trials using anti-inflammatory drugs have been disappointing, however, indicating the complexity of the problem. Two concepts that dominated the neuroinflammation field for a long time — i.e. that the CNS is 'immunologically privileged' due to a relatively impenetrable blood-brain barrier (BBB) and that inflammation necessarily exacerbates neurodegeneration — have recently been challenged by the demonstration of a substantial cross-talk between peripheral and local immune components (Rosenberg, 1997) and a contribution of numerous inflammation-associated pathways in protection against chronic neurodegenerative diseases and repair (Ekdahl et al., 2003).

Inflammation has been considered a target in the treatment of stroke (Jander et al., 2007). Brain inflammation following cerebral ischemia is believed to develop as a consequence of microglial activation, and the mobilisation and infiltration of peripheral inflammatory cells into the brain (Feuerstein et al., 1998). Cerebral ischemia induces marked inflammatory responses of resident microglia, monocytes, and macrophages, which are initiated a few hours after the onset of ischemia (Clark et al. 1994). During ischemic injury, intracellular Ca²⁺, free radicals, and hypoxia induce the expression of many pro-inflammatory genes and increase the synthesis of transcription factors such as nuclear factor-kB, interferon regulatory factor 1, and STAT3 for the regulation of inflammatory cytokine production (Salminen et al. 1995; Iadecola et al. 1999; Wen et al. 2006). The development of post-ischemic brain inflammation is co-ordinated by the activation, expression and secretion of numerous pro-inflammatory mediators from the brain parenchyma and vascular cells including cytokines, leukotriens and adhesion molecules (Giulian et al., 1993; del Zoppo 2000). Pro-inflammatory cytokines, such as tumor necrosis factor (TNFα) and interleukin (IL)-1β (Liu et al. 1994; Wang et al. 1997), and anti-inflammatory cytokines, such as IL-6 and IL-10 (Vila et al. 2003; Sotgiu et al. 2006) are released by the injured
ischemic brain cells. In response to the ischemic injury the endothelial cells express adhesion molecules including intercellular adhesion molecule 1 (ICAM-1), P-selectins, and E-selectins on their surface to attract neutrophils and macrophages (Haring et al. 1996; Lindsberg et al. 1996; Zhang et al. 1998). The hallmark of cerebral ischemia inflammation is neutrophil infiltration (Matsu et al., 1994). Neutrophils are known to release injurious mediators (Tomita and Fukuuchi, 1996). They also contribute to ROS generation ($O_2$ & $H_2O_2$) via NADPH oxidase (Ellis et al., 1989).

Blood brain barrier breakdown

The blood-brain barrier (BBB) is the specialized system of capillary endothelial cells that protects the brain from harmful substances in the blood stream, while supplying the brain with the required nutrients for proper function. Unlike peripheral capillaries that allow relatively free exchange of substance across between cells, the BBB strictly limits transport into the brain through both physical (tight junctions) and metabolic (enzymes) barriers. Thus the BBB is often the rate limiting factor in determining permeation of therapeutic drugs into the brain (Fig. 1.14). Additionally, BBB breakdown is theorized to be a key component in stroke associated pathologies (Wang et al., 2011; Michalski et al., 2010).

Brain edema

Cerebral edema is characterized by accumulation of excessive fluid in the substance of the brain. The brain is especially susceptible to injury from edema, because it is located within a confined space and cannot expand. Brain edema is also known as brain swelling, and wet brain (Fig. 1.15). Brain edema can be classified into two different types on the basis of morphological characteristics:

1. Vasogenic or “wet” edema, the result of an increased BBB permeability, and
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(2) Cytotoxic or “dry” edema, the result of the actual swelling of the cells of the brain parenchyma (Klatzo, 1967).

- Vasogenic edema is the type of edema most often present in the brain after injury, induced by ischemic stroke, brain tumors or inflammatory lesions. The BBB expresses morphological changes during the onset of vasogenic brain edema, such as the opening of tight junctions and a damaged endothelial cell membrane, followed by migration of leukocytes into the CNS (Klatzo, 1987).

- Cytotoxic brain edema is the most prominent clinical disorder after ischemic processes in the CNS and is characterized by an increase in the water content of the cells of the CNS, which may be caused by a disturbance in the transport systems for potassium and sodium rather than from changes in the permeability of the BBB.

ANTIOXIDANT SYSTEM IN THE BRAIN

The cell is able to handle and survive the continuous production of ROS because of the existence of a delegate balance between the various pro-oxidants and the antioxidant defence system. Antioxidants are exogenous (natural or synthetic) or endogenous compounds that reduce generation of ROS by acting in several ways including removal of $O_2^-$, scavenging reactive oxygen species or their precursors, inhibiting ROS formation and binding metal ions needed for catalysis of ROS generation.

Cellular antioxidant defence is classified into two categories: non-enzymatic and enzymatic (Table. III). Primary non-enzymatic antioxidant are vitamin C, vitamin E and...
ubiquinol etc, in addition thiol containing antioxidants such as reduced glutathione (GSH) which directly scavenge ROS. Enzymatic antioxidants enzymes include superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase etc. (Fig. 1.16).

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Cu-Zn SOD (cytosol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mn SOD (mitochondria)</td>
</tr>
<tr>
<td></td>
<td>GSH peroxidase</td>
</tr>
<tr>
<td></td>
<td>GSH-S- transferase</td>
</tr>
<tr>
<td></td>
<td>GSSG reductase</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
</tr>
<tr>
<td></td>
<td>Quinone reductase</td>
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</tbody>
</table>

**Repair System**

- Methionine sulfoxide reductase
- DNA repair
- Proteolysis of oxidised proteins
- Phospholipase A2 acyl transferase

**Ion sequestration**

- Transferrin
- Ferritin
- Lactoferrin
- Ceruloplasmin
- Metallothioneins

**Small molecules**

- Ascorbate
- GSH
- Bilirubin
- α- tocopherol
- Ubiquinol
- Urate
- Carotenoids

Cellular antioxidants include a wide range of systems. They act by chelating catalyst, directly scavenging free radicals or by directly detoxifying reactive oxygen species.

**Lipid peroxidation**

Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages biological membranes and generates a number of secondary products that possess neurotoxic activity (Halliwell and Chirico, 1993). Lipid
peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids i.e. those lipids containing more than two carbon-carbon double covalent bonds (Halliwell, 1992). Several experimental evidences indicate that extensive lipid peroxidation results in loss of membrane integrity, impairment of the function of membrane-transport proteins and ion channels, disruption of cellular ion homeostasis and eventual rupture leading to release of cell and organelle contents such as lysosomal hydrolytic enzymes (Fong et al., 1973; Mattson, 1998). This process proceeds by free radical chain reaction mechanism (Fig. 1.17). As with any radical reaction, these reactions consist of three major steps: initiation, propagation, and termination.

Glutathione system
The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases and glutathione S-transferases (Meister and Anderson, 1983). This system is found in animals, plants and microorganisms (Creissen et al., 1996; Meister and Anderson, 1983). The tripeptide glutathione (GSH; \( \gamma \)-L-glutamyl-L-cysteinyl-glycine) is one of the most abundant intracellular thiol in cytosol, nuclei and mitochondria (Meister, 1981; Meister et al., 1979) representing the major soluble antioxidant in these cell compartments. In the brain, the concentration of GSH is ~ 2 mM (Cooper et al., 1980; Rehncrona et al., 1980) of which 0.3% is in the oxidized form (GSSG). GSH is synthesized in cytosol by consecutive reactions of two enzymes: \( \gamma \)-glutamylcysteine (\( \gamma \)Glu-Cys) synthetase and GSH synthetase. GSH
synthesis can be limited by the ATP availability (Shan et al., 1989). In nucleus GSH maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression.

Oxidized glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Chi et al., 2005). GSH plays an important role in the protection of the cells from oxidative damage by (1) reducing disulphide groups of proteins and other cellular molecules, or by (2) scavenging free radicals and active oxygen species (McLennan et al., 1991). GSH has a major intracellular antioxidant activity being involved in detoxification of peroxides and electrophilic toxins as a substrate for GSH peroxidase and GSH transferase. GSH reacts directly with radicals in non-enzymatic reactions being the electron donor in the reduction of peroxides catalyzed by selenium-containing glutathione peroxidase (GPx). Glutathione peroxidase is the most abundant and is a very efficient scavenger of hydrogen peroxide, while it is most active with lipid hydroperoxides. Surprisingly, glutathione peroxidase is dispensable, as mice lacking this enzyme have normal lifespans, (Ho et al., 1997) but they are hypersensitive to induced oxidative stress (de Haan et al., 1998). The product of the oxidation of GSH is glutathione disulfide (GSSG). GSH is regenerated from GSSG within the cells in a reaction catalyzed by glutathione reductase (GR) (Fig. 1.18). This enzyme regenerates GSH by transferring reducing equivalent from NADPH to GSSG. NADPH regeneration during GSH redox cycling in brain depends on NADPH—regenerating enzymes such as glucose-6-phosphate dehydrogenase (G6PDH). G6PDH is a cytoplasmic enzyme that affects
the production of reduced form of cytosolic nicotinoadenosine dinucleotide phosphate coenzyme (NADPH) by controlling the step from glucose-6-phosphate to 6-phosphogluconate in the pentose phosphate pathway (Beutler et al., 1996; Kletzien et al., 1994). This enzyme is highly conserved during evolution and plays multiple roles in the cell. Until recently, the role of this housekeeping enzyme in the cell response to the oxidative stress was limited to human erythrocytes that lack any other NADPH in producing route. However, recent observations have shown that the G6PDH also plays a protective role against reactive oxygen species in eucaryotic cells that possess alternative routes for the production of NADPH and that G6PDH expression is upregulated by oxidants through a mechanism acting mainly on the rate of transcription of this gene (Cramer et al., 1995; Salvermini et al., 1999). NADPH can also be regenerated by 6-phosphogluconate dehydrogenase (6PGD) as well as malic enzyme (MEs), NADP⁺-dependent isocitrate dehydrogenases (ICDHs) and mitochondrial nicotinamide nucleotide transhydrogenase (Minich et al., 2003; Bukato et al., 1995). In addition, the glutathione S-transferases also show high activity with lipid peroxides (Sharma et al., 2004). These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Hayes et al., 2005).

Superoxide Dismutases (SODs)
The superoxide adduct is dismutated by superoxide dismutase into \( \text{H}_2\text{O}_2 \), which is converted to water and oxygen (Fig. 1.19). Based on the metal ion requirements and the anatomical distribution three major superoxide dismutases exist in brain cells: Cu, ZnSOD (SOD1), MnSOD (SOD2) and extracellular SOD (SOD3) (Table. IV). Cu, ZnSOD containing cooper and zinc at its active site is mainly found in the cytosolic and lysosomal fractions, but also in the mitochondrial intermembrane space. Mn-SOD containing manganese at its active site is located in the mitochondrial matrix. Cu, Zn-SOD and Mn-SOD are highly expressed in neurons.

It has been observed that in Mn-SOD deficit mice the neurological outcome and infarction are aggravated after both transient (Kim et al., 2002) and permanent (Murakami et al., 1998) focal ischemia. Conversely, overexpression of Mn-SOD prevented apoptosis and reduced tissue damage after focal ischemia (Keller et al., 1998). Cu, Zn-SOD overexpression in adult mouse brains reduces the tissue damage.
after transient focal ischemia (Saito et al., 2003) but in the immature brain Cu, Zn-SOD overexpression, increases the tissue damage after hypoxia-ischemia was observed (Ditelberg et al., 1996). This was attributed to limited capacity of immature brain to convert the $\text{H}_2\text{O}_2$ into water and $\text{O}_2$ because of the lower levels of catalase and glutathione peroxidase (Fullerton et al., 1998). The third SOD isoform (EC-SOD) is present in extracellular fluids such as plasma and is also expressed in the

Table IV Mammalian Superoxide dismutase (adapted from Niizuma et al., 2010)

<table>
<thead>
<tr>
<th>Mammalian superoxide dismutases</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>SOD1</strong></td>
</tr>
<tr>
<td><strong>(Cu,ZnSOD)</strong></td>
</tr>
<tr>
<td><strong>Location</strong></td>
</tr>
<tr>
<td>Cytosol</td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
</tr>
<tr>
<td>32,000</td>
</tr>
<tr>
<td><strong>Structure</strong></td>
</tr>
<tr>
<td>Dimer</td>
</tr>
<tr>
<td><strong>Metals, g-atomic/sulphate</strong></td>
</tr>
<tr>
<td>Cu 1, Zn 1</td>
</tr>
<tr>
<td><strong>Phenotype of transgenic mouse</strong></td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td><strong>Phenotype of knockout mutant</strong></td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td><strong>Chromosome</strong></td>
</tr>
<tr>
<td>11 (rat)</td>
</tr>
</tbody>
</table>

| **SOD2**                       |
| **(MnSOD)**                    |
| **Location**                    |
| Mitochondria                   |
| **Molecular weight**            |
| 63,000                          |
| **Structure**                   |
| Tetramer                        |
| **Metals, g-atomic/sulphate**   |
| Mn 1                            |
| **Phenotype of transgenic mouse** |
| Normal                          |
| **Phenotype of knockout mutant** |
| Normal                          |
| **Chromosome**                  |
| 16 (mouse)                     |

| **SOD3**                       |
| **(ECSOD)**                    |
| **Location**                    |
| Extracellular space             |
| **Molecular weight**            |
| 120,000                         |
| **Structure**                   |
| Tetramer                        |
| **Metals, g-atomic/sulphate**   |
| Cu 1, Zn 1                      |
| **Phenotype of transgenic mouse** |
| Normal                          |
| **Phenotype of knockout mutant** |
| Normal                          |
| **Chromosome**                  |
| 5 (mouse)                      |

Cu,Zn, copper, zinc; Mn, manganese; EC, extracellular.
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brain but at lower levels than Cu, ZnSOD and MnSOD (Marklund et al., 1982). In adult focal ischemia, EC-SOD overexpression conferred protection (Fukui et al., 2002) and EC-SOD deficiency aggravated the injury.

Catalase
The defense of the brain cells against peroxide mediated oxidative damage is essential for maintaining functionality of the brain. Catalase and the glutathione system are both important for cellular detoxification of H$_2$O$_2$ (Dringer et al., 1999; Liddell et al., 2004). Under physiological conditions, catalase accepts only H$_2$O$_2$ as substrate but not organic hydroperoxides (Aebi et al., 1984). The heme-containing enzyme converts H$_2$O$_2$ to H$_2$O and O$_2$. Catalase is a diffusion-controlled enzyme and therefore is especially effective when the clearance of high concentration of H$_2$O$_2$ is required. It has been observed that in cultured neurons the disposal rate for H$_2$O$_2$ is strongly reduced by inhibition of catalase, demonstrating the role of this enzyme in the neuronal clearance of exogenously applied H$_2$O$_2$ (Sokolova et al., 2001).

Nitric oxide synthases
Since the original suggestion that nitric oxide synthesis plays a role in cerebral ischemia (Marshall and Kontos, 1990) over 800 research reports have addressed this issue. Nitric oxide is enzymatically synthesized from L-arginine and is massively increased by ischemia (Wei et al., 1999). Three nitric oxide synthases (NOS) have been reported (eNOS, nNOS and iNOS), so named because of their originally defined endothelial (eNOS) and neuronal (nNOS) localization, or ability to be upregulated when induced (iNOS). Nitric oxide may also serve as an antioxidant against products of the Fenton reaction. At the same time, iNOS expression has been implicated as a critical factor for promoting postischemic neurogenesis (Zhu et al., 2003). Further, iNOS expression may contribute to increased tolerance of brain to ischemia induced by preconditioning stimuli (Kapinya et al., 2002) as does eNOS upregulation (Hashiguchi et al., 2004). The fact that eNOS and nNOS are Ca$^{2+}$ dependent, while iNOS is not, can be used to distinguish among them for mechanistic purposes. The relevance of nitric oxide was increased with the report that the diffusion limited reaction between superoxide and nitric oxide gives rise to peroxynitrite (Beckman et al., 1990). The highly reactive peroxynitrite provided a mechanistic basis for oxidative stress derived from increased nitric oxide production.
caused by ischemia/reperfusion (Eliasson et al., 1999). Nitric oxide has also been shown to inhibit mitochondrial respiration via competition with oxygen for cytochrome oxidase (Brown and Borutaite, 1999) and play a role in the initiation of apoptosis (Bonsfoco et al., 1995). Although little has been reported on efforts to bring nitric oxide inhibitors to clinical investigation, there is no doubt that nitric oxide plays a pivotal role in mediating oxidative stress (Mikkelsen and Wardman, 2003).

**DNA damage activated poly (ADP-ribose) polymerase (PARP)**

Poly (ADP-ribose) polymerase is a nuclear enzyme that is strongly activated by single stranded DNA breaks; there are no other known activators (Berger, 1985). NAD is an essential cell survival factor that participates in various critical cellular processes, including energy metabolism. ADP-ribose cyclase synthesis, and class III histone deacetylase (Ying, 2006). NAD also acts as the substrate of poly (ADP-ribose) polymerase1 (PARP-1), which once activated, catalyzes transfer of ADP-ribose moieties from NAD to target proteins. The literature shows that cerebral ischemia/reperfusion results in PARP-1 over activation and consequent decline of NAD levels in the brain (Eliasson et al., 1999; Endres et al., 1997). As NAD is essential for the mitochondrial electron transport reaction, NAD depletion is thought to suppress mitochondrial function and ATP generation, leading to the release of apoptosis inducing factor (AIF) and eventually cell death (Yu et al., 2002).

In non-proliferating cells such as neurons, however, NAD is highly compartmentalized, and the mitochondrial pool of NAD is not readily depleted by PARP-1 activation (Ying et al., 2005). Thus, neuronal NAD depletion is most likely to menace cell survival by repressing other NAD-dependent signalling pathways (Ying et al., 2005; Pillai et al., 2005) (Fig.1.20).

Oxidative DNA damage is a severe consequence of oxidative stress that, if not repaired, results in cell death via activation of several pathways (Li et al., 2011).
Endogenous oxidative damage to nuclear DNA that produces base damage, apurinic/apyrimidinic abasic site (AP sites), and strand breaks occurs rapidly after cerebral ischemia/reperfusion (Fujimura et al., 1999) and is an important trigger of ischemic cell death (Chen et al., 1997). In the brain, DNA base excision repair (BER) functions as the major repair mechanism for oxidative DNA damage and neuronal BER activity is highly regulated after ischemia and reperfusion. Although BER activity is markedly upregulated in the brain after sublethal insults (Lan et al., 2003; Lin et al., 2000) including the neuroprotective paradigm of ischemic preconditioning (Li et al., 2006), it rapidly declines after lethal ischemic injury, leading to the accumulation of cytotoxic oxidative DNA lesions (Lan et al., 2003; Li et al., 2006).

Drugs used in our studies
Hesperidin
Synonyms
Hesperetin 7-rhamnoglucoside
Hesperitin-7-rutinoside

Properties
Empirical Formula \( C_{62}H_{44}O_{13} \)
Molecular Weight 610.56
MP 250-255 °C

Description
Hesperidin a member of the flavanone group of flavanoids (Fig. 1.21) is aglycone form is called hesperetin. The phytochemical hesperidin is mainly found in citrus fruits such as lemons and oranges. The highest concentration of hesperidin can be found in the white parts and pulps of the citrus peels. Hesperidin is well known to posses antioxidant, anti-inflammatory, hypolipidemic, vasoprotective and anticarcinogenic and cholesterol lowering properties (Galati et al., 1994; Wacker and Eilmes, 1975). A number of researchers have documented the antioxidant activity and radical scavenging properties of hesperidin (Praga et al., 1987). Hesperidin is effectively used as a supplemental agent and sternohyoid muscles parallel to helps to
reduce edema or excess swelling in the legs due to fluid accumulation. The neuroprotective efficacy of hesperidin is attributed to its ability of inhibiting Fe^{2+}-induced linoleate peroxidation and auto-oxidation of cerebral membranes (Saija et al., 1995), scavenging peroxynitrite radicals (Kim et al., 2004) and inhibition of ROS generation, including hydroxyl radical (Jung et al., 2003). It protects the neurons against various types of insults associated with many neurodegenerative diseases (Kumar and Kumar, 2010). Preventive effect of hesperidin (50mg/kg) is well known in case of Huntington’s disease induced by 3-nitropropionic acid (Kumar and Kumar, 2010). Hesperidin may have a beneficial role against ACN-induced oxidative stress in the brain (El-Sayed et al., 2008). Hesperidin is also known to inhibit following enzymes: phospholipase A2, lipoxygenase, HMG-CoA reductase and cyclooxygenase (Hirata et al., 2005; Monforte et al., 1995). Hesperidin is known to be partly deglycosylated in the gut to its aglycone hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone) by intestinal microflora (Kim et al., 1998).

Silymarin

**Synonyms**

\[(2,3-\text{Dihydro}-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-1,4-benzodioxin-6-yl)-2,3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one\]

**Properties**

- **Empirical Formula**: C_{22}H_{23}O_{19}
- **Molecular Weight**: 482.44
- **MP**: 230 - 233 °C

**Description**

Silymarin, a flavonoid is a member of the Asteraceae family (Compositae), is extracted from the seeds of milk thistle (*Silybum marianum*) and is known to own antioxidant potential (Singal et al., 2010) and anti-inflammatory property (Manna et al., 1999) (Fig. 1.22). Silymarin has been reported to decrease lipid peroxidation (Bosisio et al., 1992). Furthermore, it has been demonstrated that its antioxidative activity is related to the scavenging of free radicals (Rauen et al., 1998) and activation
of antioxidative defenses; increases in cellular glutathione (GSH) content (Valenzuela et al., 1989) and superoxide dismutase activities (Muzes et al., 1991). Silymarin pretreatment is known to inhibit inflammation via inhibiting IkB-α degradation and NF-κB nuclear translocation in ischemic brain tissues (Hou et al., 2010). Silymarin shows poor solubility in water which led to the development of enhanced formulations. There have also been prepared glycosides of silybin, which show better water solubility and have stronger hepatoprotective effect (Kosina et al., 2002). Silibinin, a main component of silymarin is seems to show protection against oxidative stress as it has been reported to decrease lipid peroxidation, a sensitive marker of oxidative lipids, in liver microsomes and isolated hepatocytes (Bosisio et al., 1992) and lipopolysaccharide-induced neurotoxicity (Wang et al., 2002). Silymarin is known to have protective properties against skin cancer and anti-atherosclerotic (inhibits expression of adhesion molecules). In a recent study it is well demonstrated that silymarin reduces maneb and paraquat induced Parkinson’s disease phenotype (Singhal et al., 2011). Another report confirm that silymarin attenuates the amyloid β plaque burden and improve behavioral abnormalities in an Alzheimer’s disease mouse model (Murata et al., 2010). Silymarin is also known to have enhancing learning memory deficit induce by ethanol toxicity (Neese et al., 2004).

Naringenin

Synonyms

2,3-Dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one
4',5,7-Trihydroxyflavanone.

Properties

Empirical Formula \( C_{15}H_{12}O_5 \)

Molecular weight 272.25

MP 247-250 °C

Fig. 1.24 Structure of Naringenin

Description

Naringenin (Fig. 1.24) is present in grapefruits (Citrus paradisi) and kino fruit (Eucaliptus maculata) mainly as its glycosylated form, naringin. Many biological functions of naringenin have been studied, including anti-inflammatory, antioxidative
free radical scavenging and lipid peroxidation inhibitory effect and carbohydrate metabolism promoter (Limasset et al., 1993; Younes et al., 1981), antimutagenic (Calomme et al., 1996) and anticarcinogenic effects (So et al., 1996). The anti-inflammatory effects of naringenin have been well documented, but the mechanism is poorly characterized. Naringenin has also been shown to reduce oxidative damage to DNA in-vitro. Naringenin has been shown to have an inhibitory effect on the human cytochrome P450 isoform CYP1A2, which can change pharmacokinetics in a human (or orthologous) host of several popular drugs in an adverse manner, even resulting in carcinogens of otherwise harmless substances (Edward and Bernier, 1996). Naringenin has also been shown to reduce hepatitis C virus production by infected hepatocytes (liver cells) in cell culture. This seems to be secondary to Naringenin ability to inhibit the secretion of very-low-density lipoprotein by the cells (Nahmias et al., 2008). The flavanone naringenin and its glycosides are widespread in nature, and can reach significant concentrations in commonly consumed citrus juices (Mouly et al., 1994). A recent study revealed that naringenin could be a potential immunomodulator for inhibiting lung fibrosis (Du et al., 2009) and metastasis and so, could be an ideal therapeutic agent in the treatment of both cancer and fibrosis.

Ellagic Acid

**Synonyms** 4, 4', 5, 5', 6, 6'-Hexahydroxydiphenic acid 2, 6, 2', 6'-dilactone

**Properties**

**Empirical Formula** $C_{14}H_{14}O_8$

**Molecular weight** 302.19

Ellagic acid (Fig. 1.25) is a natural phenol antioxidant found in numerous fruits and vegetables, rich in strawberries but 50% more in raspberries (mainly ellagitannins). The antioxidant and anti-proliferative properties of ellagic acid have spurred preliminary research into the potential health benefits of ellagic acid consumption (Han et al., 2006; Hagiwara et al., 2010). Ellagic acid has antioxidant properties in a number of in vitro and small-animal models presumably by acting as a scavenger of oxygen species produced by hydrogen peroxide treatment, and as a protector of the DNA double helix from alkylating agent injury (Seeram et
al., 2005). It is a well known inhibitor of glutathione-S-transferase (Das et al., 1984). Used for the assay of factor XIIa in plasma (Dooijewaard and Kluft, 1983) and contact activation in blood coagulation (Lxner et al., 1982). The antiproliferative properties of ellagic acid are due to its ability to directly inhibit the DNA binding of certain carcinogens, including nitrosamines (Mandal et al., 1990) and polycyclic aromatic hydrocarbons. These properties have generated interest in potential human health benefits from the consumption of ellagic acid. However, till now very little study of these proposed benefits has been reported. A small randomized controlled trial involving 19 patients with carotid artery stenosis found that pomegranate juice, which is high in ellagic acid, appeared to reduce blood pressure and carotid artery wall thickness (Aviram et al., 2004).